Construction of a highly thermostable 1,3-1,4-β-glucanase by combinational mutagenesis and its potential application in the brewing industry

Section in which the paper is to be considered: Microbial and Enzyme Technology

Construction of a highly thermostable 1,3-1,4-β-glucanase by combinational mutagenesis and its potential application in the brewing industry

Chengtuo Niu[a,b,c] Linjiang Zhu[a,b] Annie Hill[c] R. Alex Speers[c] Qi Li[a,b,*]

[a] Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

[b] Lab of Brewing Science and Technology, School of Biotechnology, Jiangnan University, Wuxi 214122, China

[c] International Center for Brewing and Distilling, Heriot-Watt University, Edinburgh EH14 4AS, United Kingdom

*Correspondence to: Qi Li, School of Biotechnology, Jiangnan University, 1800 Lihu Avenue, Wuxi, Jiangsu, China 214122 . E-mail: liqi@jiangnan.edu.cn

Tel: +86-510-85918176. Fax: +86-510-85805219.

Abstract

Objectives

To improve the thermostability and catalytic property of a mesophilic 1,3-1,4-β-glucanase by combinational mutagenesis and to test its effect in congress mashing.

Results

The mutant β-glucanase (rE-BglTO) constructed by combinational mutagenesis showed a 25°C increase in optimal temperature, a 19.5°C rise in T_{50} value and a 15.6°C increase in melting temperature (T_m) compared to wild-type enzyme. Its half-life values at 60°C and 70°C were 151.9 min and 98.8 min, which were 367% and 798% higher than those of wild-type enzyme. Besides, its specific activity and k_{cat} value were 4273.7 U mg^{-1} and 188.6 s^{-1} while its stability under acidic conditions was also improved. In flask fermentation, the catalytic activity of rE-BglTO reached 2380.9 U ml^{-1}, which was 62.9% higher than that of wild-type enzyme. The addition of rE-BglTO in congress mashing decreased the filtration time and viscosity by 21.3% and 9.6%, respectively.

Conclusions

The mutant β-glucanase showed high catalytic activity and thermostability which indicated that rE-BglTO is a good candidate for application in the brewing industry.

Key words: 1,3-1,4-β-glucanase; catalytic property; combinational mutagenesis; congress mashing; thermostability

Introduction

1,3-1,4-β-glucanases can hydrolyze β-glucans into oligosaccharides by cleaving the 1,4-β-glycosidic bonds in a strict manner (Planas 2000). In the brewing industry uses cereals as raw materials and β-glucanases with high catalytic efficiency and thermostability are required to degrade the β-glucans in the cereal cell walls. This increases extract yields and reduces filtration times in mashing (Chaari et al. 2014) and reduces the chance of haze development in packaging (Jin et al. 2004). The optimal temperatures of *Bacillus* β-glucanases are usually around 45-65ºC, which are not able to withstand the heat environment in industry (Planas 2000). Though some thermophilic β-glucanases from thermophiles can hold their optimal temperatures around 80ºC (Schimming et al. 1991), their catalytic activities are not suitable for application under industrial conditions with relatively lower temperatures. Besides, the catalytic activities of most β-glucanases from wild-type microbes are usually low (Yang et al. 2008). Therefore, enhancing the thermostability and catalytic activity of mesophilic β-glucanases is required.

To enhance the thermostability of β-glucanase, various enzyme engineering strategies, such as directed evolution (Mao et al. 2016) and hybridization (Olsen et al. 1991), were adopted. However, more thermostable β-glucanases are required to meet brewing standard. In addition to thermostability, the yield of β-glucanases from wild-type microbes is usually low, even with process optimization and control (Yang et al. 2008). Therefore, improving the yield of recombinant β-glucanase is important for its application in industry. A combination of thermostability beneficial mutations was reported to be able to further improve the protein thermostability (Zhang et al. 2010). In our previous researches, the thermostability of a mesophilic β-glucanase gene (*bglT*) from *Bacillus terquilensis* CGX 5-2 was improved by lysine-based site-directed mutagenesis (Niu et al. 2015), disulfide bond engineering (Niu

et al. 2016) and site-saturation mutagenesis, respectively. In order ~~T~~to further enhance its thermostability, the above beneficial sites were combinational mutated within a mesophilic β-glucanase (BglT) in this study. The optimized gene (*rE-BglTO*)~~–~~ was constructed by gene synthesis and expressed in *Escherichia coli* BL21(DE3). Its thermostability and catalytic properties were characterized and compared to wild-type enzyme. The effect of rE-BglTO in Congress mashing was determined and compared to commercial enzymes.

Materials and Methods

~~Clone~~Cloning, expression and purification of β-glucanase in *E. coli*

The *rE-BglTO* gene which harbored the eleven beneficial mutation, including K20S, N31C, S40E, S43E, E46P, P102C, K117S, N125C, K165S, T187C and H205P, was synthesized (the amino acid sequences of the *BglT* gene and *rE-BglTO* gene were shown in Supplementary Figure 1) and ligated into vector~~recombinant plasmid~~ **pUC57**~~-*rE-BglTO* synthesized~~ by Genewiz (Suzhou, China). The recombinant plasmid **pUC57**-*rE-BglTO* was digested by the restriction enzymes *Bam*HI and *Xho*I, ligated into vector **pET28a**(+) and transformed into *E. coli* BL21(DE3) competent cells. The positive clones were verified by DNA sequencing analysis. The recombinant *E. coli* cells were cultivated in optimized TB media (20g yeast extract/l; 12.5 g tryptone/l; 14.1 ml glycerol/l; 2.17 g KH_2PO_4/l and 2.74 g K_2HPO_4/l) at 37°C. The expression and purification of recombinant β-glucanase were conducted through a Ni-NTA affinity column (Qiagen) according to previous reported methods (Niu et al. 2015). The purity of enzyme was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was estimated by the Bradford method with bovine serum albumin (BSA) as standard.

Activity assay of 1,3-1,4-β-glucanase

The activity of 1,3-1,4-β-glucanase was measured using the improved AZO method (Niu et al. 2014) using 1% AZO barley β-glucan (Megazyme, Wicklow, Ireland) as the substrate. The reducing sugar content was measured using the 3,5-dinitrosalicylic acid (DNS) method. One unit of the 1,3-1,4-β-glucanase activity was defined as the amount of enzyme that released 1 μmol reducing sugar from the substrate per minute at pH 6.5. Each measurement was repeated in triplicate. The kinetic parameters of β-glucanase were determined at 40°C and estimated by Eadie-Hofstee plots.

Effects of temperature, pH, metal ions and inhibitors on enzymatic activity and stability

The optimal temperature and pH were determined by measuring the enzymatic activities at a range of parameters while the other conditions remained constant. The pH stability and thermostability were conducted according to a previous reported method (Niu et al. 2016). The midpoint temperature (T_{50}) value was defined as the temperature at which β-glucanase lost half of its activity after treatment at 40-90°C for 10 min. The half-life value of β-glucanase, designated as $t_{(1/2,X^{o}C)}$, was defined the time that the β-glucanase activity decreased to 50% at set temperatures. The melting temperature (T_m), $\triangle H$ and $\triangle G$ values were determined by a Q2000 differential scanning calorimeter (DSC) (TA, New castle, USA) at a protein concentration of 1 mg ml^{-1} in 20 mM phosphate buffer (pH6.5).

To estimate the effect of metal ions and inhibitors on enzyme activity, the purified β-glucanase was incubated in different metal ion solutions, including KCl, NaCl, $CaCl_2$, $MgCl_2$, $FeCl_2$, $FeCl_3$, $CoCl_2$, $ZnCl_2$, $MnCl_2$ and $CuCl_2$ (1 mM and 10 mM) and inhibitor solutions, including EDTA, DTT and β-mercaptoethanol (1 mM and 10 mM) at 40°C for 1 h. After that, the residual activities were measured and compared with the activities of enzyme without any treatment.

Far-UV circular dichroism (CD) analysis and 3D structure analysis

The far-UV spectra of β-glucanases was measured from 190 nm to 250 nm by a MOS-45 circular

dichroism spectrometer (Bio-logic, Claix, France). The proportions of secondary structure elements (helix, β-sheet, β-turn and random coil) were estimated by the Dichroism online server (Whitmore and Wallace 2008). The 3D structures of the wild-type BglT and rE-BglTO were homologically modeled in SWISS-MODEL online server with the 3D structure of β-glucanase from *B.subtilis* (PDB code: 3O5S) as template. The amino acid sequence identities between them were 99.53% and 94.37%, respectively.

Effects of β-glucanase on the filtration time and viscosity of mash

Pilsner malt (20 g) was ground to grind 7 in a DLFU disc mill (Bühler Miag, London, UK) and suspended in 100 ml of pre-heated water (45ºC). This mash was started with the addition of 1.0 ml wild-type BglT, rE-BglTO or a commercial enzyme (Ultraflo®L, Novozymes, Denmark) through EBC standard Congress mashing process (the mashing temperature was maintained at 45ºC for 30 min, then raised to 70ºC at a rate of 1ºC min^{-1} and added 100 mL water in the mash) (4.5.1 Analytical-EBC 1997). A similar control with 1 ml water instead of enzyme solution was used. The filtration time was determined by filtering 20 ml cooled mash through a quantitative filter paper (Grade 113V, GE Healthcare, Freiburg, Germany) while the filtration of mash without enzyme treatment was used as control. The reduction of filtration time was calculated using the following equation:

$$\Delta\psi = (\psi_{control}-\psi)\times 100/\psi_{control}$$

where ψ and $\psi_{control}$ are the total flow time of mash with or without enzyme treatment and $\Delta\psi$ is the filtration time reduction.

The mash viscosity was determined at 20ºC by Wells-Brookfield Cone/Plate digital viscometer (Brookfield AMETEK, MA, USA) with Cone CP-40 at a shear rate of 90 s^{-1} (Speers et al. 2004). The viscosity reduction was calculated using the following equation:

$\Delta\mu = (\mu_{control} - \mu) \times 100/\mu_{control}$

where μ and $\mu_{control}$ are the viscosity of mash with or without enzyme treatment and $\Delta\mu$ is viscosity reduction.

Results and discussion

Production of recombinant β-glucanase

The optimized gene *rE-BglTO* which harbored K20S, N31C, S40E, S43E, E46P, P102C, K117S, N125C, K165S, ~~T187S~~ T187C and H205P mutation sites was synthesized, ligated into **pET28a**(+) vector and transformed into *E.coli* BL21(DE3) competent cells. Fig.1a showed that the growth curve of recombinant *E.coli* harboring *rE-BglTO* gene was ~~almost the same with~~similar to that of recombinant *E. coli* harboring *BglT* gene. After 9 h shaking at 37°C, the dry cell weight values of the recombinant *E. coli* cells harboring *rE-BglTO* and *BglT* gene were ~~both around~~ 2.~~7~~883 g l^{-1} and 2.814 g l^{-1}, respectively, which were slightly higher than that of wild-type *E.coli* cells (2.514 g L^{-1}). The recombinant *E. coli* cells began to secrete β-glucanases when the inducers were added into the media. Fig.1b showed that the highest enzymatic activity of rE-BglTO was 2380.9 U ml^{-1} when induced ~~for~~ at 6 h, which was 62.9% higher than that of wild-type BglT (1461.3 U ml^{-1}).

Figure 1

The recombinant wild-type BglT and rE-BglTO were then purified and verified by SDS-PAGE analysis (Supplementary Figure ~~1~~2). The catalytic properties of the wild-type BglT and rE-BglTO were determined and compared (Table 1). The specific activity of rE-BglTO was 4273.7 U mg^{-1}, which was 71.6% higher than that of the wild-type BglT (2490.1 U mg^{-1}). The K_m value for rE-BglTO (0.273 mM) was slightly lower than that of the wild-type BglT (0.297 mM), which indicated that rE-BglTO had better binding affinity with substrate barley β-glucan than the wild-type BglT. Besides, the k_{cat} and k_{cat}/K_m values of rE-BglTO were 188.6 s^{-1} and 690.8 s^{-1} mM^{-1}, respectively, which were 37.3% and 49.4%

Table 1

higher than those of the wild-type BglT. These results indicated that the combination of beneficial mutations could greatly enhance the catalytic efficiency and binding affinity of β-glucanase.

Effects of temperature and pH on stability of β-glucanase

As shown in Fig.2a, the optimal temperature of rE-BglTO was 70°C, which was 25°C higher than that of the wild-type BglT (45°C). rE-BglTO could maintain more than 80% activity between 35-70°C, while the wild-type BglT could only maintain high activity between 35-55°C. rE-BglTO also showed an improvement in kinetic stability, since its T_{50} value was 18.5°C higher than that of the wild-type BglT (Fig.2b). The changes in enzyme kinetic stability was further confirmed by the half-life values. Fig.2c showed that the wild-type BglT was almost fully inactivated after incubation at 60°C for 140 min while rE-BglTO still could maintain around 60% activity after the same treatment. At 70°C, the wild-type BglT was soon inactivated while rE-BglTO still could have more than 60% activity left after 80 min treatment (Fig.2d). The half-life values of rE-BglTO at 60°C and 70°C were 151.9 min and 98.8 min, respectively, which were 367% and 798% higher than those of the wild-type BglT (32.5 min and 11 min). As for enzyme thermodynamic stability, the T_m value of rE-BglTO was 55.9°C, which was 15.6°C higher than that of the wild-type BglT (Table 2). DSC method also revealed some thermodynamic stability related parameters. As shown in Table 2, the $\triangle H$ value of rE-BglTO was much higher than that of the wild-type BglT, which indicated that more energy was required for denaturation of rE-BglTO. The $\triangle G$ value for rE-BglTO was 4.4 kcal mol^{-1} higher than that for the wild-type BglT. The kinetic and thermodynamic parameters of rE-BglTO were also higher than those of mutants previously reported (Fig.2 and Table 2), which ~~These results~~ indicated that the combinational mutagenesis could greatly enhance the kinetic and thermodynamic stability of β-glucanase.

Figure 2
Table 2

The optimal pH value of rE-BglTO was shifted from pH6.5 to pH6.0 (Fig.3). It also showed better

Figure 3

stability at acidic environments. There were still 74.8% and 92% activities left for rE-BglTO after one hour incubation at pH4.5 and pH5.5 while the remaining activities for the wild-type BglT were 7.9% and 59.9%, respectively. This could improve the performance of rE-BglTO in mashing between mash is a weakly acidic solution (pH5-5.5).

Effects of metal ions and inhibitors on stability of β-glucanase

Metal ions and chemical reagents have been shown to play key roles in protein folding and catalysis (Andreini et al. 2008). The effect of various cations and inhibitors on the activity of the wild-type BglT and rE-BglTO was tested. As shown in Table 3, the presence of Fe^{2+} (10 mM) and Fe^{3+} (10 mM) both strongly inhibited the activities of the wild-type BglT (8.4% and 6.5%) and rE-BglTO (10.4% and 3.5), respectively, while the enzymes were moderately inhibited by Ca^{2+} (84.2% and 86.4%), Co^{2+} (93.9% and 94.7%), Li^{2+} (51.3% and 52.0%), Cu^{2+} (86.2% and 89.7%) and Zn^{2+} (47.6% and 43.6%) when the cations were present at 10 mM. Meanwhile, the presence of Cu^{2+} could greatly activate the activities of the wild-type BglT and rE-BglTO to 167.4% and 169.4% at 1 mM. The presence of Mn^{2+} could enhance the rE-BglTO activity to 123.9% at 1 mM while the activity of the wild-type BglT was moderately inhibited to 80.4%. The activities of both enzymes were almost uninfluenced by K^+, Na^+ and $NH4^+$. Among the inhibitors, the enzymatic activities of the wild-type BglT and rE-BglTO were both greatly inhibited by DTT (34.6% and 30.6%), EDTA (30.9% and 16.7%) and β-mercaptoethanol (40.9% and 30.3%) at 10 mM.

Table 3

Effect of β-glucanase on the filtration time and viscosity of mash

The filtration time and viscosity of mash after treatment with the wild-type BglT and rE-BglTO were measured and compared with a commercial β-glucanase. As shown in Table 4, the addition of the

Table 4

wild-type BglT, rE-BglTO and the commercial enzyme reduced the mash filtration time by 8.4%, 21.3% and 16.8%, respectively. The wild-type BglT, rE-BglTO and the commercial enzyme also reduced the mash viscosity by 3.5%, 9.6% and 7.9%. This indicated that the performance of rE-BglTO in β-glucan degradation in mashing was superior to the wild-type BglT and the commercial enzyme.

Conformational changes in molecular structures of β-glucanase

To understand the mechanism for the thermostability improvement, the secondary structures of the wild-type BglT and rE-BglTO were analyzed by CD. Fig.4 showed that both the positive and negative bands in CD spectrum of rE-BglTO shifted to larger wavelengths compared to the wild-type BglT. This indicated that rE-BglTO might had more β-sheet structures, which was confirmed by the secondary structure percentage analysis (Supplementary Table 1). The proportions of helix, β-sheet and β-turn in rE-BglTO were increased by 1%, 12% and 5% compared to the wild-type BglT, respectively, while the percentage of random coil was decreased from 39% to 21%. The results of secondary structures were further confirmed by the 3D structures of wild-type BglT and rE-BglTO. Fig.5 showed that the 3D structures of the wild-type BglT and rE-BglTO was both the identical ~~anti-parallel β-sheet jellyroll~~β-sandwich structure with anti-parallel β-sheets. However, several different places were observed. Two helix structures α1 (residues No.36-38) and α2 (residues No.97-99) were lost in rE-BglTO while two new helix structures ~~(~~α 3 (residues No.140-142) and α4 (residues No.190-193) were formed. Moreover, three new β-sheet structures β1 (residues No.6-8), β2 (residues No.18-20) and β3 (residues No.60-66) were formed while eight β-sheet structures were prolonged (residues No.52 in β4, –No.~~73~~72-73 in β5, No.92-95 in β6, No.104-107 in β7, No.122-124 in β8, No.150-152 in β9, No.178-180 in β10 and –No.211-213 in β11). More structured residues protein structure could result in more rigid protein overall and local structure, which might be the reason for the enhancement of

Figure 4

Figure 5

enzyme thermostability (Di Marino et al. 2014). Several residues related to β-glucanase substrate binding, such as Glu63, Arg65, Phe92, Tyr94, Glu105, Asn121 and Tyr123, were also located in the transformed regions. The reason for the enhancement of catalytic properties might be the large-scale secondary structure changes in the substrate binding region, especially Glu105 which was reported to be the key catalytic nucleophile in β-glucanase catalysis process (Planas 2000). More negative surface charge was observed in rE-BglTO compared to the wild-type BglT (Supplementary Figure ~~2~~3). This might be the reason for the shift of optimal pH and better stability in acidic environment since acidophilic enzymes were reported to contain more acidic residues on their surface (Michaux et al. 2010).

Conclusion

In this study, a highly thermostable 1,3-1,4-β-glucanase (rE-BglTO) was constructed by combination of eleven beneficial mutations. The optimal temperature and melting temperature of rE-BglTO were 25°C and 15.6°C higher than those of the wild-type BglT. Its half-life values at 60°C and 70°C were 367% and 798% higher than those of the wild-type BglT. The catalytic activity of rE-BglTO could reach 2380.9 U ml^{-1}, which was 62.9% higher than that of the wild-type BglT. The mutant enzyme also showed good ability to reduce the viscosity and filtration time of mash, indicating its potential value for application in the brewing industry.

Acknowledgement

The authors appreciated Prof. Wilfred J Mitchell (School of Life Sciences, Heriot-Watt University) for providing lab space and equipment. The authors are also grateful to Mrs. Vicky Goodfellow (International Centre for Brewing and Distilling, Heriot-Watt University) for her help in congress mash preparation and analysis. This study was financially supported by the National High

Technology Research and Development Program 863 (No.2013AA102106), National Science Foundation (No.31271919, No.31571942 & No.31301539), and the Fundamental Research Funds for the Central Universities (JUSRP51504, JUSRP51402A & JUDCF13008), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), Program of Introducing Talents of Discipline to Universities (No.111-2-06).

Supporting information

Supplementary Fig.1-Amino acid sequences of the *BglT* gene and *rE-BglTO* gene.

Supplementary Fig.~~1~~2-SDS-PAGE analysis of purified the wild-type and recombinant 1,3-1,4-β-glucanases.

Supplementary Table 1-Comparison of secondary structure between the wild-type BglT and rE-BglTO using Dichroweb online software.

Supplementary Fig.~~2~~3-Comparison of electrostatic surface potential of the wild-type BglT and rE-BglTO.

References

Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM (2008) Metal ions in biological catalysis: from enzyme databases to general principles. J Biol Inorg Chem 13:1205-1218. doi: 10.1007/s00775-008-0404-5

Chaari F, Belghith-Fendri L, Blibech M, Driss D, Ellouzi SZ, Ellouz-Chaabouni S (2014) Biochemical characterization of a lichenase from Penicillium occitanis Pol6 and its potential application in the brewing industry. Process Biochem 49:1040-1046. doi: 10.1016/j.procbio.2014.02.023

Di Marino D, Achsel T, Lacoux C, Falconi M, Bagni C (2014) Molecular dynamics simulations show how the FMRP Ile304Asn mutation destabilizes the KH2 domain structure and affects its function. J Biomol Struct Dyn 32:337-350. doi: 10.1080/07391102.2013.768552

Jin YL, Speers A, Paulson AT, Stewart RJ (2004) Effects of β-glucans and environmental factors on the viscosities of wort and beer. J Inst Brew 110:104-116. doi: 10.1002/j.2050-0416.2004.tb00189.x

Mao S, Gao P, Lu Z, Lu F, Zhang C, Zhao H, Bie X (2016) Engineering of a thermostable β-1,3-1,4-glucanase from Bacillus altitudinis YC-9 to improve its catalytic efficiency. J Sci Food Agr 96:109-115. doi: 10.1002/jsfa.7066

Michaux C, Pouyez J, Mayard A, Vandurm P, Housen I, Wouters J (2010) Structural insights into the acidophilic pH adaptation of a novel endo-1,4-β-xylanase from Scytalidium acidophilum. Biochimie 92:1407-1415. doi: 10.1016/j.biochi.2010.07.003

Niu C, Zhu L, Wang J, Li Q (2014) Simultaneous enhanced catalytic activity and thermostability of a 1,3-1,4-β-glucanase from Bacillus amyloliqueformis by chemical modification of lysine residues. Biotechnol Lett 36:2453-2460. doi: 10.1007/s10529-014-1616-0

Niu C, Zhu L, Xu X, Li Q (2016) Rational design of disulfide bonds increases thermostability of a mesophilic 1,3-1,4-β-glucanase from Bacillus terquilensis. Plos One 11:e0154036. doi: 10.1371/journal.pone.0154036

Niu C, Zhu L, Zhu P, Li Q (2015) Lysine-based site-directed mutagenesis increased rigid β-sheet structure and thermostability of mesophilic 1,3-1,4-β-glucanase. J Agr Food Chem 63:5249-5256. doi: 10.1021/acs.jafc.5b00480

Olsen O, Borriss R, Simon O, Thomsen KK (1991) Hybrid Bacillus (1-3,1-4)-β-glucanases: engineering thermostable enzymes by construction of hybrid genes. Mol Gen Genet 225:177-185. doi: 10.1007/BF00269845

Planas A (2000) Bacterial 1 3-1,4-β-glucanases: structure, function and protein engineering. BBA-Protein Struct Mol Enzymol 1543:361-382. doi: 10.1016/S0167-4838(00)00231-4

Schimming S, Schwarz WH, Staudenbauer WL (1991) Properties of a thermoactive β-1,3-1,4-glucanase (lichenase) from Clostridium thermocellum expressed in Escherichia coli. Biochem Biophys Res Commun 177:447-452. doi: 10.1016/0006-291X(91)92004-4

Speers R, Patelakis S, Paulson A, Oonsivilai R (2004) Shear rates during brewing operations. Tech Quart-Master Brewer Assn Am 41:241-247.

Whitmore L, Wallace BA (2008) Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. Biopolymers 89:392-400. doi: 10.1002/bip.20853

Yang S, Qiaojuan Y, Jiang Z, Fan G, Wang L (2008) Biochemical characterization of a novel thermostable β-1,3-1,4-glucanase (lichenase) from Paecilomyces thermophila. J Agr Food Chem 56:5345-5351. doi: 10.1021/jf800303b

293 Zhang Z-G, Yi Z-L, Pei X-Q, Wu Z-L (2010) Improving the thermostability of Geobacillus
294 stearothermophilus xylanase XT6 by directed evolution and site-directed mutagenesis.
295 Bioresource Technol 101:9272-9278. doi: 10.1016/j.biortech.2010.07.060

Table 1 Catalytic properties of the wild-type BglT, K20S/K117S/K165S mutant, N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO

Enzymes	Specific activity (U mg^{-1})	K_m (mM)[a]	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
BglT	2490.1±55.3	0.297±0.005	137.4±1.9	462.5±19.1
K20S/K117S/K165S	3936.4±39.6	0.278±0.004	184.9±2.7	665.2±19.7
N31C-T187C/P102C-N125C	4045.4±47.9	0.277±0.004	186.3±2.2	672.6±17.7
E46P/S43E/H205P/S40E	4093.8±61.1	0.271±0.003	187.4±1.7	691.5±13.6
rE-BglTO	4273.7±26.9	0.273±0.003	188.6±1.3	690.8±12.5

[a] The K_m value was calculated according to the Eadie-Hofstee plots. The enzyme concentration was 100 μg ml^{-1} while the substrate concentration varied from 1 to 10 mg ml^{-1}. The experiments were repeated in triplicate

Table 2 Thermodynamic stability parameters of the wild-type BglT, K20S/K117S/K165S mutant, N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO. The protein melting temperature (T_m) and the thermodynamic stability parameters were determined by a Q2000 differential scanning calorimeter (DSC) (TA, New castle, USA) at a protein concentration of 1 mg ml^{-1} in 20 mM phosphate buffer (pH6.5)

Enzymes	T_m (°C)	△H (kcal mol^{-1})	△G (kcal mol^{-1})
WT	40.3	173.5	6.3
K20S/K117S/K165S	50.8	189.4	8.8
N31C-T187C/P102C-N125C	54.9	207.6	10.3
E46P/S43E/H205P/S40E	54.1	204.7	10.1
rE-BglTO	55.9	205.1	10.7

Table 3 Effect of metal ions and inhibitors on activities of the wild-type BglT and rE-BglTO (CK are the activities of β-glucanase samples with same amount of buffer solution instead of the metal ion or inhibitors)

Metal ions/inhibitors	Relative activity of wild-type BglT (%)		Relative activity of rE-BglTO (%)	
	1 mM	10 mM	1 mM	10 mM
CK	100	100	100	100
$CaCl_2$	75.3±1	84.2±1.2	83.1±1.0	86.4±0.8
$CoCl_2$	83.8±0.7	93.9±1.2	81.6±0.9	94.7±1.3
$CuCl_2$	167.4±1.1	86.2±0.9	169.4±1.3	89.7±0.7
$FeCl_2$	44.4±0.8	8.4±0.1	48.7±1.2	10.4±0.7
$FeCl_3$	28.7±0.3	6.5±1.1	19.8±0.3	3.5±0.2
KCl	101.3±0.9	99.9±0.3	100.7±0.6	100.1±0.4
$LiCl_2$	82.1±0.8	51.3±1.4	84.9±0.7	52.0±1.1
$MgCl_2$	189.4±0.6	50.0±0.5	189.6±0.7	53.4±1.1
$MnCl_2$	80.4±1.1	55.2±0.6	123.9±1.2	63.9±0.8
NH_4Cl	98.7±0.5	100.3±0.4	100.3±0.3	102.1±0.6
NaCl	100.3±0.7	97.8±1.1	101.3±0.9	98.3±1.3
$ZnCl_2$	55.8±1.2	47.6±0.4	50.9±0.6	43.6±0.3
DTT	79.8±0.7	34.6±0.9	73.1±0.7	30.6±0.7
EDTA	71.3±1.6	30.9±0.8	39.6±0.2	16.7±0.9
β- mercaptoethanol	73.1±1.2	40.9±1.6	68.7±1.0	30.3±0.8

311 Table 4 Effect of the wild-type BglT, rE-BglTO and a commercial β-glucanase (Ultraflo® L) on the

312 viscosity and filtration time of mash

Samples	Filtration time (s)	Filtration time reduction (%)	Viscosity (mPa.s)	Viscosity reduction (%)
Control	202±3	-	1.14±0.01	-
BglT	185±3	8.4	1.10±0.01	3.5
rE-BglTO	159±2	21.3	1.03±0.01	9.6
Commercial enzyme	168±2	16.8	1.05±0.01	7.9

313

Figure legends

Fig.1 The growth curves (a) and enzymatic activity curves (b) of the wild-type *E. coli*, recombinant *E. coli* harboring the *BglT* and *rE-BglTO* genes. The growth curves were determined by cultivation of *E. coli* cells at 37°C, 200 rpm. The enzymatic activities curves were determined by measuring the enzymatic activities at ~~40°C~~ different time points since IPTG and α-lactose were added into the media. The data was presented as mean±standard deviation from three independent experiments

Fig.2 The optimal temperature curves (a), kinetic stability curves (b), the inactivation curves at 60°C (c) and the inactivation curves at 70°C (d) of the wild-type BglT, K20S/K117S/K165S mutant, N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO. The optimal temperature curves was determined by measuring the enzymatic activities at a temperature range of 45°C to 80°C with 5°C as interval. The kinetic stability curves was determined by measuring the enzymatic activity after treatment from 40°C to 80°C for 10 min and following 10 min on ice. The inactivation curves were determined by incubation the enzymes at 60°C and 70°C and enzymatic activities were measured at a set of time points. Error bars ~~indicated~~ were obtained from the standard deviation from triplicates. 100% activities of the wild-type BglT, K20S/K117S/K165S mutant, N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO were 1530.2 U ml^{-1}, 1897.5 U ml^{-1}, 1903.4 U ml^{-1}, 2515.7 U ml^{-1} and 2950.3 U ml^{-1}

Fig.3 The optimal pH and pH stability of the wild-type BglT, K20S/K117S/K165S mutant, N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO. (a) The optimal pH of the wild-type BglT-, K20S/K117S/K165S mutant, N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO were determined by measuring the enzymatic activities in acetic acid/sodium acetate buffer (pH4.0-6.0) and sodium phosphate buffer (pH6.0-8.5) at 40°C; (b)

The pH stability was calculated by dividing the residue activities of enzymes after 1 h treatment in different pH buffers by enzymatic activities without any treatment. The data plotted were averages of triplicate experiments. 100% activities of the wild-type BglT, K20S/K117S/K165S mutant, N31C-T187C/P102C-N125C mutant, E46P/S43E/H205P/S40E mutant and rE-BglTO were 1461.3 U ml^{-1}, 1220.1 U ml^{-1}, 1363.5 U ml^{-1}, 2103.8 U ml^{-1} and 2380.9 U ml^{-1}

Fig.4 The CD spectrums of the wild-type BglT and rE-BglTO in 20 mM sodium phosphate buffer (pH6.5). The Far-UV CD spectrums were measured from 190 nm to 250 nm in a 0.1 cm path length quartz cell with a resolution of 1 nm and corrected by subtracting the proper baseline. The concentrations of β-glucanases were both 100 μg ml^{-1}

Fig.5 Comparison of the 3D structures of the wild-type BglT and rE-BglTO. (a) the concave side of the wild-type BglT; (b) the concave side of rE-BglTO; (c) the convex side of the wild-type BglT; (d) the convex side of rE-BglTO. The helix, β-sheet and loop structures were colored red, yellow and green, respectively. The calcium ion was shown in sphere (green dot). The eleven mutation sites were shown in sticks and labeled. The labeled α and β represented the α-helix and β-sheet structures, respectively